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13. ABSTRACT ***(Maximum 200 Words)***

This research project is designed to further our knowledge on the neural basis of alcohol addiction. Alcohol consumption by humans can be modeled in the rat using operant behavioral procedures in which an arbitrary response, such as a lever press, is reinforced by the presentation of small amounts of orally consumed ethanol solutions; this paradigm is termed ethanol self-administration. In the studies in this project simultaneous recording of ensembles of individual neurons during ethanol self-administration is used to determine in real-time the contribution of patterns of neural activity to ongoing alcohol-seeking behavior. The contribution of excitatory and dopaminergic inputs to the alcohol-associated neuronal activity in the accumbens, a brain region that is an integral part of the neural circuitry of addiction, is being tested. Thus far, we have confirmed the feasibility of a reversible inactivation technique used to study the afferents to the accumbens (Objective 1), and have begun studies on the effect of abstinence on accumbens neural activity (Objective 3). These studies will provide information on how the neurons of the accumbens control alcohol-seeking behavior during times when alcohol is available and during abstinence.

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Introduction

This annual report summarizes the accomplishments to date for the first year of this research project. This research project is designed to further our knowledge on the neural basis of alcohol addiction. Alcohol consumption by humans can be modeled in the rat using standard operant behavioral procedures in which an arbitrary response, such as a lever press, is reinforced by the presentation of small amounts of ethanol solutions that can be orally consumed; this paradigm is termed ethanol self-administration. Recent advances in behavioral neurophysiology allow for the simultaneous recording of ensembles of individual neurons during ethanol self-administration permitting us to determine in real-time the contribution of patterns of neural activity to ongoing alcohol-seeking behavior. The nucleus accumbens is a brain region that is an integral part of the neural circuitry of addiction, including alcohol addiction. By recording neuronal activity within this brain region during ethanol consumption, we can begin to understand how this region participates in alcohol-seeking behavior. Previously, we characterized the primary neuronal activity patterns that occur in the nucleus accumbens (NA) during ethanol self-administration. The studies within this proposal are designed to determine the afferent regions that induce these behavioral correlates, with the hypothesis that different behavioral correlates recorded within the NA are induced by activity within distinct afferents, such as the amygdala and the frontal cortices. The modulation of these responses by the dopaminergic inputs from the ventral tegmental area (VTA) will also be tested. In addition, we propose to extend our results examining the neural activity patterns of the NA within a model of relapse to alcohol consumption after a period of abstinence. The elucidation of the neural mechanisms that underlie the production of ethanol-seeking behavior is essential for an understanding of the biological basis of alcohol addiction, and will contribute to the development of therapies to treat alcohol abuse in humans.

Body

This research project contains 4 objectives within the statement of work. Two of those objectives, Objective 1 and 3, were slated to begin in Year 1; the remaining two were slated to begin in Year 3.

Objective 1. Studies within Objective 1 proposed to test the dependence of behavior-related neurophysiological correlates in the nucleus accumbens on activity of specific afferents to the accumbens, namely the prefrontal cortex (PFC), the amygdala, and the orbitofrontal cortex (OF). For these experiments, we proposed to reversibly inactivate the afferent region to determine how the electrophysiological correlates, and ethanol self-administration behavior, were altered. This project has begun by examining the effects of inactivation of the PFC. These studies are ongoing, and so final conclusions are not possible. However, our initial pilot studies have allowed us to demonstrate that unilateral PFC infusion of muscimol does not alter behavior, hence we can study the effects of "shutting down" afferents to the accumbens, whilst still having behavior around which to examine the neural activity. Further we have proof that muscimol infusion does inhibit activity at the infusion site. We have tested this by implanting recording electrodes alongside infusion cannulae. We found that muscimol infusion depresses PFC activity within 15 minutes after infusion if the tip of the cannula is placed 1-2 mm above the tips of the electrodes; in contrast if the tip of the cannula is less than 1 mm above or even below the tips of the electrodes, the onset of inhibition is much later, likely reflecting the slow spread of the drug back upwards towards the electrode tips. Hence we now know that 1) muscimol infusion does depress neural activity, at least within the PFC; and that 2) infusion cannulae must be slightly above electrode tips if we plan to record at the infusion site. Examples of these pilot data are found in Figure 1.

Progress on this objective is occurring slower than anticipated because I have yet to fill one position, that of the postdoctoral researcher. However, I am happy to report that I have identified a candidate for this position, who will arrive in January 2003. Progress should greatly increase after that time.

Objective 3. Studies in Objective 3 were proposed to determine the neural activity patterns that occur in the nucleus accumbens during extinction and reinstatement of ethanol self-administration. Previously, we had described the neural activity within the accumbens during ethanol self-administration by rats. As described under Objective 3,

our current studies are examining the changes in this neural activity during a period of abstinence when the ethanol is no longer available, although the subjects are still actively seeking the ethanol. We find that neural activity within the nucleus accumbens (and its afferent regions) changes rapidly under conditions of extinction, when reward is no longer available. This is true if one examines single-unit firing patterns, or the local field potentials (see Figure 2) recorded from the same wires. Specifically, certain neural firing patterns are present only when the reward itself is being delivered, although the animal's behavior (lever-pressing, and then checking in the reward delivery report for the presence of the reward) is the same in general during conditions when the reward is and isn't available.

Importantly, these studies are still in progress (i.e., we have only collected data from a subset of the total subjects) and were originally slated to continue through the end of Year 2. Hence final conclusions shall be presented in subsequent reports. However, we already can say that these findings show that the contribution that the accumbens makes in guiding alcohol-seeking behavior changes under conditions when alcohol is not available, a situation analogous to the abstinent human. In addition, these results are in agreement with our previous investigation of extinction in rats responding for the natural reward, sucrose, and further strengthen the notion that alcohol-seeking usurps brain circuits that normally are recruited to guide animals towards biologically-significant rewards in the environment.

Key Research Accomplishments

1. Initiation of studies under Objective 1 have determined that muscimol infusion depresses neural activity near the site of the infusion; hence this technique can be used to study reversibly inactivate afferent regions to the accumbens.
2. Identification of changes in accumbens neural activity when ethanol is no longer available, indicating that this brain region is exquisitely sensitive to the presence and absence of the ethanol reward.

Reportable Outcomes

Studies conducted under this award have resulted in the following manuscripts and abstracts:

1. Janak, P.H. (2002) Application of Many-Neuron Microelectrode Array Recording to the Study of Reward-Seeking Behavior. *Methods Drug Abuse: Cellular and Circuit Level Analysis* (ed. By B. Waterhouse), CRC Press, Boca Raton, *in press.*

2. I. Zironi and P.H. Janak (2002) Neuronal ensemble and field potential recordings in rat hippocampus and nucleus accumbens during a self-administration paradigm: context as a reinstatement cue. *FENS Abstract, in press.*

Conclusions

Our studies for Objective 1, just underway, indicate that the technique is feasible, and so we anticipate completing this Objective in the next reporting period. The work in progress for Objective 3 indicates that the accumbens plays a different role in guiding alcohol-seeking behavior under conditions when alcohol is not available, a situation analogous to the abstinent human. Different contributions of neurons in the accumbens to alcohol-seeking behavior when it is available, and during abstinence, when it is not available, indicate that unique therapies may be indicated for human alcoholics that should depend upon whether or not they are abstinent. In addition, these results are in agreement with our previous investigation of extinction in rats responding for the natural reward, sucrose, and further strengthen the notion that alcohol-seeking usurps brain circuits that normally are recruited to guide animals towards biologically-significant rewards in the environment.

Appendices

1. Figure 1.

2. Figure 2.

3. Zironi and Janak, 2002.

4. Janak, 2002.

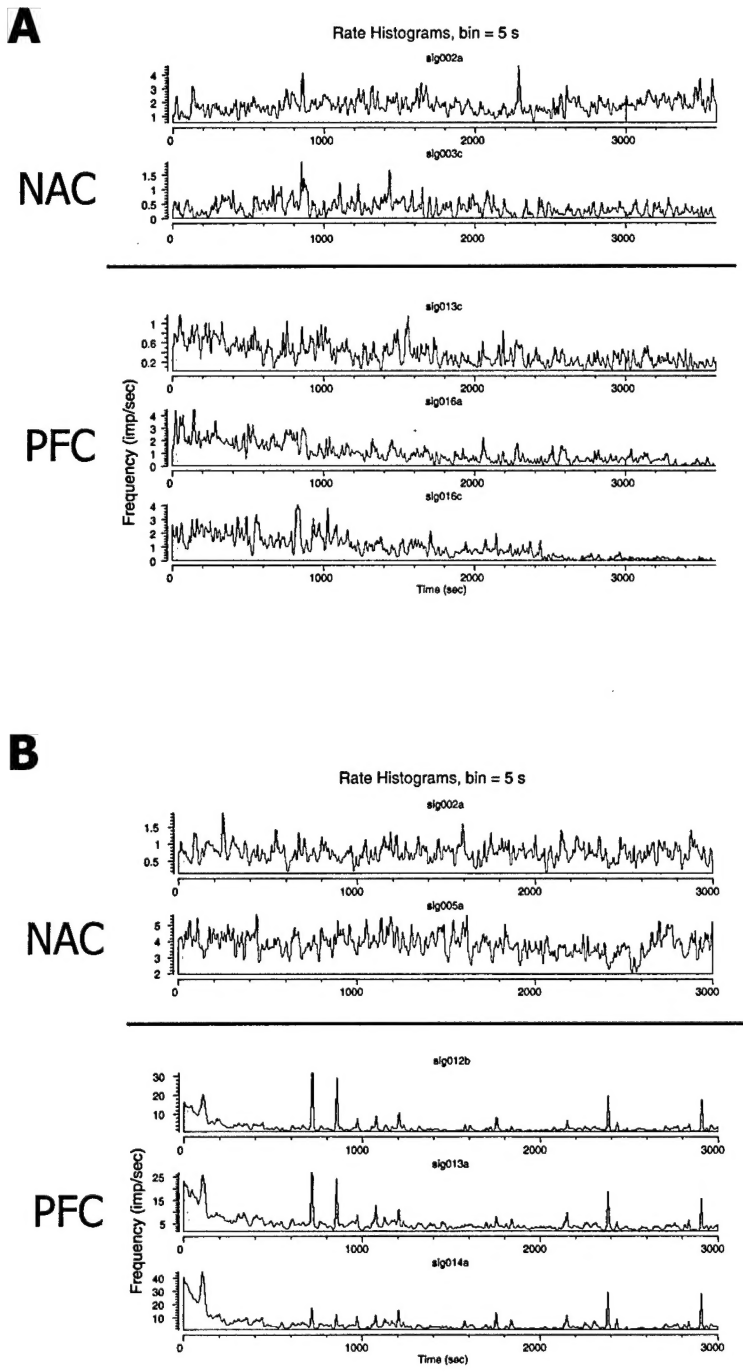

Figure 1. Rate histograms of a selection of single units recorded from the nucleus accumbens (NAC) and the prefrontal cortex (PFC) of awake rats. Recordings began 10 minutes after infusion of 0.5 ul of 0.1 ug/ul muscimol. **A**. In this subject (G8 4/12/02), muscimol infusion decreased firing rates of PFC, but not NAC, units slowly throughout the 1-hour recording session. **B**. For this subject (G7, 4/14/02), muscimol infusion decreased firing rates of PFC, but not NAC, units rapidly, within the first 5 minutes of the session. An overall decrease in firing was apparent for the remainder of the session.

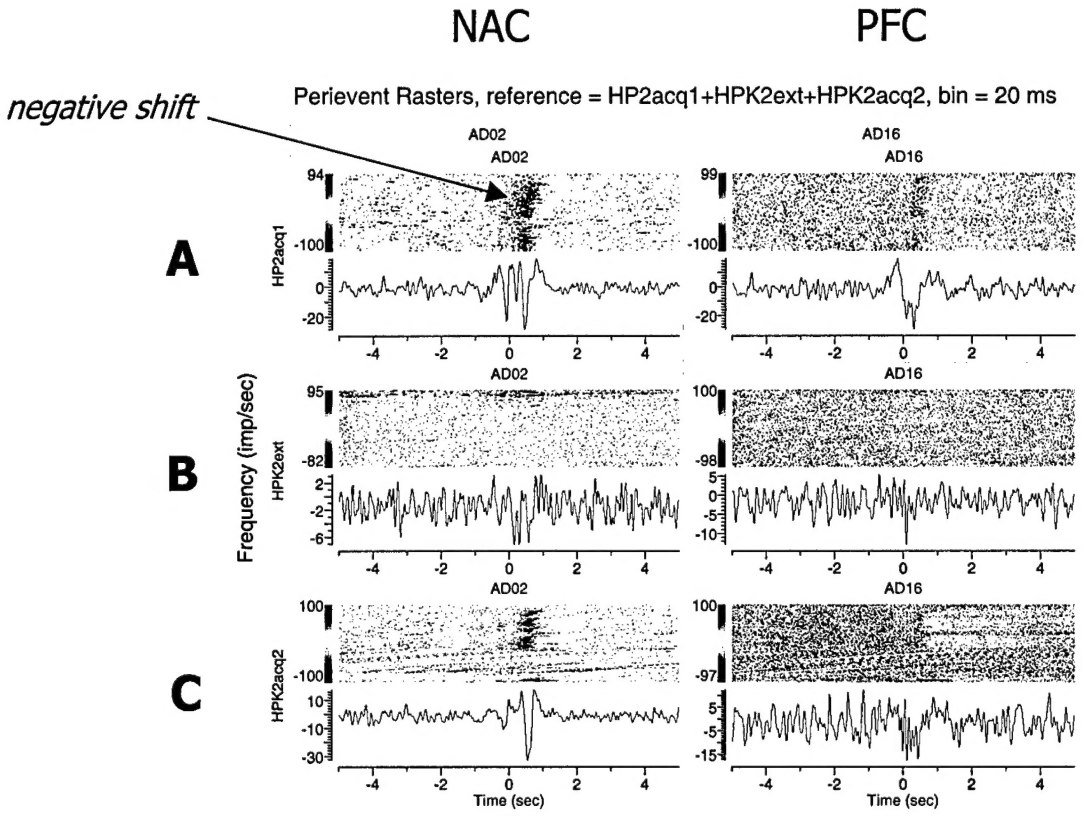

Figure 2. Perievent raster of local field potentials (LFP) recorded from the nucleus accumbens (NAC) and the prefrontal cortex (PFC) during a within-session extinction-reinstatement procedure. The average LFP relative to the time when the subject inserts snout into the reward receptacle (t=0 on the x-axis) is shown in the lower half of each panel, while the individual trials are shown in the raster in the top half of each figure, with positive an negative intensity of the field conveyed via color coding. **A.** When reward is offered following successful completion of the FR1 lever press schedule, a prominent negative shift in the NAC and PRC LFPs is observed just after the subject inserts his snout into the reward receptacle. **B.** Negative shift rapidly disappears within the first few trials of extinction, when the reward is no longer offered following the operant response. **C.** Partial recovery of the negative shift in both the NAC and the PFC is observed after the reward is again available following an operant response. Bin size = 20 ms. Subject G8.

NEURONAL ENSEMBLE AND FIELD POTENTIAL RECORDINGS IN RAT HIPPOCAMPUS AND NUCLEUS ACCUMBENS DURING A SELF-ADMINISTRATION PARADIGM: CONTEXT AS A REINSTATEMENT CUE.

I. Zironi and P.H. Janak

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The hippocampus (HIPP) induces the depolarised "up" state of nucleus accumbens (NACC) medium spiny neurons (Goto & O'Donnel, J. Neurosci. 21, 2001), thereby driving NACC neuronal activity. If this is true, then the HIPP should contribute to reward-seeking behavior mediated by the NACC. We investigated the physiological relationship between the HIPP and NACC during the acquisition, extinction and reinstatement of operant responding for either sucrose or ethanol in a paradigm that manipulates context. Extracellular single units and local field potentials (LFP) were recorded from the HIPP and NACC of rats trained to bar press for sucrose or ethanol in Context A. A negative shift in the NACC LFP appeared during acquisition that was temporally correlated to the animal approaching the reward port. Following extinction in Context B, the NACC reward-related LFP negative shift disappeared. When animals were tested for context-dependent non-reinforced reinstatement of responding in the Context A, bar pressing significantly increased relative to extinction baseline. The NACC reward-related negative shift in the LFP did not re-emerge until the reward was again delivered. Decreases in the firing rate correlated to reward approach were observed in a subpopulation of NACC units during the acquisition phase, and when reward was again delivered following reinstatement, but not during extinction. Correlational analyses revealed a high probability of temporally-related firing between the HIPP and the NACC during reward-seeking behavior. These results indicate that context can provide a cue for reinstatement of responding for reward, that synchronous activity in the NAC reflects the presence of reward, and that a physiological relationship between HIPP and NACC activity can be detected during reward-seeking behavior.

Application of Many-Neuron Microelectrode Array Recording to the Study of Reward-Seeking Behavior

Patricia H. Janak

Contents

1. Introduction

This chapter describes the application of multichannel electrophysiological recording in the awake behaving rat to the study of the neurobiology of drug abuse and reward. The *in vivo* many-neuron recording technique described here allows for the simultaneous measurement of the spike activity of dozens of individual neurons in behaving rodents. Its purpose is to provide a real-time window onto neuronal function so that we may better understand the relationship between neuronal activity and behavior. Many-neuron electrophysiology therefore can be used to ask questions regarding the contribution of specific neural circuits to reward-seeking behavior. We can ask: how do neuronal networks acquire, process, and act upon information about rewards in their environment?

The advantages of the chronic many-neuron recording technique are three-fold. First, because many neurons are recorded at the same time, the data collection rate is faster than previous one-neuron-at-a-time methods of recording neurons during behavior. This makes neuron recording practical for use in conjunction with rat behavioral models. Second, because neurons within the same and across different brain regions are recorded simultaneously, we can begin to understand how neurons work together, within and across brain regions, to accomplish complex behaviors. Third, we can begin to define with greater precision the contribution of a particular brain region to a particular behavior on the actual millisecond time scale used by the brain for interneuronal communication. This type of temporal precision has contributed to research on the role of the nucleus accumbens in drug self-administration as described by Peoples (this volume) on nucleus accumbens neural activity during intravenous cocaine self-administration by rats. This

chapter will continue the discussion of the technique as applied in our laboratory to other, orally-delivered, reinforcers, and will consider some of the issues relevant to the analysis of many-neuron spike train data.

2. Description of the Technique

2.1. Electrodes

The *in vivo* many-neuron recording technique described here is designed to allow for the simultaneous detection of the spike activity of dozens of individual neurons in behaving rodents. The first important component of this technique is the electrode, which measures local changes in the extracellular potential field produced when a neuron whose cell body is near the tip of an electrode emits an action potential. The amplitude and shape of the recorded waveform depend upon a variety of factors, such as the size and shape of the neuron, including its dendritic tree, and the relative distance between the electrode tip and the cell body (Lemon & Prochazka, 1984). Because we are recording extracellular potentials, this technique does not allow exact identification of the recorded cell type in the manner of intracellular techniques, which allow one to fill the cell with dye to facilitate later identification. Hence, the first issue to be aware of is that cell-type identification can only be made using characteristic electrophysiological features that have been identified from intracellular recording experiments, such as firing rates and bursting patterns.

Many types of electrodes have been used for recording multiple units, including microwires and glass micropipettes. Electrodes made of metal microwires are obviously much less delicate than glass electrodes filled with conducting solutions and therefore are

more convenient for use in behaving rats. Recently, silicon and ceramic electrodes with multisite recording circuits have been etched using thin-film photofabrication technology (c.f., Bragin et al., 2000). In theory, the silicon and/or ceramic probe technology should allow for chronic *in vivo* recordings with an increase in the number of neurons one can record and less concomitant disruption of neural tissue. So far successful long-term recordings in behaving animals with this technology has remained elusive (http://www.engin.umich.edu/facility/cnct/backind.html; Moxon, 1999). Hence, the best choice to date for electrodes for chronic implant appears to be microwire arrays. These arrays provide reliable and long-lasting signal acquisition in behaving subjects. The arrays or bundles of wire are inserted into the brain regions of interest, and are then repeatedly attached to preamplifiers, digitizers, and computers for acquisition of neural data. Microwire electrode arrays are typically made of insulated stainless steel, tungsten, nichrome (nickel-chrome alloy) or platinum. Microwire diameters in the range of 25-80 microns work well for detecting extracellular potentials of one or more nearby cell bodies. Fine beveled tips on these wires are sometimes used, although it is now clear that blunt cut wires are easier to prepare and are sufficient for recording extracellular spikes.

Electrode bundles or arrays may be homemade or bought commercially. The electrode arrays we use are available from NBLabs (Dennison, TX). They are constructed from Teflon-insulated 50 micron stainless steel wire soldered to plastic female connectors. These electrode arrays are arranged in groups of 8 or 16 wires, with one stainless steel or silver ground wire per 8 recording electrodes. An in-depth discussion of electrodes used in chronic ensemble recording in behaving animals can be found in a recent review by Moxon ((Moxon, 1999). The insertion of microwire arrays

into brain is described below, followed by a description of the data acquisition hardware and software.

2.2. Chronic Implants of Microelectrode Arrays

The quality and longevity of many-neuron chronic recordings appear to depend upon the creation of a secure headstage (Williams et al., 1999; Janak, in press). Here we describe the surgical procedure for implanting the microwire electrode arrays, from NB labs or elsewhere, in some detail. The surgery can be lengthy (3-5 hours). Therefore it is important to keep the subject warm using a heating pad and to choose an anesthetic that is appropriate for long surgeries. We use a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture or isoflurane. Aseptic surgical techniques are used. The scalp is shaved and is cleaned with alcohol pads and disinfectant soap. The subject is mounted within a standard stereotaxic apparatus (David Kopf, Tujunga, CA) and antibiotic optical ointment is gently applied to the surface of each eye with a sterile cotton-tipped applicator. This ointment is reapplied every hour or so to keep the membranes of the eye moist and to protect the eye from accidental contamination with dental acrylic, etc., during the procedure.

Next, a midline scalp incision is made with a sterile scalpel and the skull surface is cleaned of blood and tissue. A dissecting microscope, or other means to magnify the surgical field, aids greatly in the remaining steps of this procedure. Hand-held or mounted drills are used to create openings in the skull large enough to accomodate the wires of the electrode arrays. The electrode arrays are secured to the stereotaxic device using holders made of male connectors that complement the female connectors of the electrode arrays. NBLabs coats their electrode arrays with an inert agent (polyethylene

glycol) along the length of the array, leaving a customer-specified bare length of wire, to enable these fine wires to penetrate the brain. Care must be taken to keep the insulation intact along the length of the wires. Because the 50-μm wires sometimes have difficulty piercing the dura, we gently rip an opening in this layer of the meninges using fine forceps. If bleeding occurs during the removal of the skull or the tearing of the dura, the region is carefully rinsed and cleaned and the electrodes are not lowered until the bleeding has ceased. The electrodes can be lowered to the desired depth over a period of minutes using micromanipulators; it is thought that slow penetration of the brain tissue produces less tissue damage, and, hence, better recordings.

Once the electrodes are in place, very small fragments of gel foam (Pharmacia and UpJohn, Kalamazoo, MI) are placed gently around the electrodes to protect the surface of the brain; the electrodes are then are secured to the skull using a small amount of dental acrylic (Jet dental acrylic, Lang Dental Mfg. Co., Inc., Wheeling, IL). Before any dental acrylic is applied, the skull is carefully cleaned and dried. A very clean and very dry skull surface is crucial for the close and tight bonding of the dental acrylic with the skull. All initial applications of dental acrylic are runny allowing the acrylic to flow easily between the individual electrodes, increasing the likelihood that the wires will be well-anchored to the cement. After the acrylic dries, the stiffening agent can be rinsed from the distal section of the array with sterile saline or PBS if desired. This process is repeated if multiple arrays are to be implanted. At least 4 skull screws are used to anchor the cement headstage to the skull. The use of many skull screws around the perimeter of the surgical area adds to the stability of the headset. Additional holes are drilled for

ground wires that are inserted 1-2 mm into cortex and are secured using a small amount of dental acrylic.

After all electrodes and ground wires are secured, and all screws have been placed, the connectors of the electrodes are arranged above the rat's skull in the desired final position using homemade holders secured to the stereotaxic device. Again, the skull is carefully cleaned and dried. Dental acrylic is then applied in stages over the entire assemblage such that all metal and the bottom half of the connectors is covered. Care is taken to remove any dental acrylic that may have seeped over the subject's skin and to ensure that no sharp edges remain that may irritate the skin. If the original skin incision is longer than the final headset requires, then sutures are used to close the tissue. Topical anesthetics (5% lidocaine ointment) are applied to the wound margin to reduce post-surgical discomfort. Topical antibiotics are used to combat infection. Injection of systemic antibiotics may also aid greatly in reducing the incidence of infection that contributes to premature loss of the dental cement headstage.

2.3. Recording Procedures

Subjects are allowed to recover from the surgery for one week before recording sessions commence. Extracellular signals from the implanted wires are detected by attaching a headset cable to the connector on the top of the rat's head. Commercial headset (recording) cables are available from NBLabs and Plexon Inc. (Dallas, TX); cables can also be made in the laboratory (Kubie et al., 1997). Headset cables contain one channel for each microwire, as well as one or more channels for ground wires, and an input for delivering power to the headset amplifiers. An important function of these cables is to boost the current of the relatively low-voltage signals detected by microwires;

this is accomplished with Field Effect Transistors (FETs) or Operational Amplifiers (Op-Amps) embedded in the headset as close to the connector as possible. The FETs or Op-Amps within the headset cables also serve to reduce the impedance of the signal output, and hence, movement artifacts generated from cable movements (Kubie et al., 1997). The opposite end of the headset/recording cable is then connected to a commutator (Dragonfly Inc., Ridgeley, WV) to allow continuous signal transmission from each channel during the subjects' movement.

Both the acquisition and the analysis of many-neuron ensemble recordings have been aided immensely by advances in electronics, including computing power and data storage capabilities. Several companies supply recording hardware and software (see (Sameshima & Baccala, 1999), for review of basic attributes of multichannel extracellular spike recording equipment). The commercial products used to collect and analyze data in our laboratory are produced by Plexon, Inc. and by Biographics, Inc. (Winston-Salem, NC). The specific attributes of each of these systems are available from the manufacturers; however, the basic requirements for any multichannel recording system are similar.

First, after passing through the commutator at the top of the behavioral chamber, the signals from each channel must be amplified, filtered and digitized. Some recording systems, such as the Plexon recording system, allow for differential recording of the signals from each wire relative to a user-selected wire (one with little or no spike activity) to further reduce the background noise measured on the wires of interest.

The ability to sample and digitize information from many independent channels simultaneously at a high rate allows us to observe a faithful picture of the actual spike

output of large distributed networks of neurons throughout the brain. This is a major advantage of multichannel recording systems, and rates from 25 – 40 kHz can be obtained with currently available commercial devices.

Data acquisition software allows for spike sorting to be carried out by the user on-line. Typically, one sorts the spikes from each channel by separating those waveforms thought to represent firing from individual neurons from each other and from the noise, and then one begins recording the neural data. On-line spike sorting routines apply statistical algorithms to assist in classifying waveforms as belonging to one or more distinct neurons. These techniques include template matching and principal component analysis, among others, as described by Wheeler (Wheeler & Heetderks, 1982). We use these techniques as implemented in the Plexon data acquisition software, as well as the intuitively simpler method of sorting using time-voltage parameters which, in this system, is accomplished visually by the placement of two adjustable windows on segments of the waveform display.

The digitized waveforms can then be saved to the hard drive of the host computer. In addition, all times of occurrence of each individual neuron's extracellular action potential, or spike, are saved. These times are referred to as timestamps and the progression of these spikes through time is commonly referred to as the spike train. At the same time, behavioral data can be collected. Many systems are now designed to interface with any behavioral control system that can emit TTL pulses for each behavioral event, such as lever presses, tones, etc. Hence the times of each spike from each user-identified neuron and the times of each behavioral event are all saved together in the same data file as separate variables, greatly simplifying off-line analysis.

The raw data files can be analyzed using homemade programs. However, software programs designed to import data files from various data acquisition systems are available, including Stranger (Biographics, Inc.) and NeuroExplorer (Plexon, Inc.). These programs rapidly prepare numerical and graphical results for many-neuron data including overall firing rates, autocorrleograms, crosscorrelograms, perievent histograms, and more. The numerical results can be exported into other software programs as needed for statistical analyses and other manipulations.

Following the final recording session, the subject is deeply anesthetized, and the location of the tips of the electrodes is marked by passing a 10-20 μA current for 10 seconds through one or more wires per array to deposit iron ions into the surrounding tissue. Subjects are then transcardially perfused with PBS followed by a 4% paraformaldehyde/3% potassium ferrocyanide solution. The potassium ferrous cyanide forms a blue reaction product with the deposited iron. Standard histological procedures are then used to slice and stain the tissue to visualize electrode location.

3. Yields, Stability, and Longevity of Recordings

The many-neuron microwire array recording technique discussed here allows for the spike activity of groups of individual neurons to be recorded simultaneously in the awake behaving rat (Woodward et al., 1998). The number of neurons one records depends upon the number of wires implanted into the brain, which determines the number of data channels required. Some wires detect the waveforms from one or more nearby neurons, while other wires are silent. Typical yields from a 16-wire implant into the dorsal or ventral striatum can vary from 5 to 20 clearly discernable units. Once subjects have

recovered from the surgery, daily recording sessions can commence. A purchased or handmade recording headset cable, described previously, is easily attached to an awake rat, and neural activity is then gathered during the behavior of interest, such as the operant self-administration of drug.

The practical advantages offered by this technique include the ability to obtain data from more than one neuron at a time, and the ability to obtain data for many days. Both of these points are likely to depend on the quality of the surgery. Placement of the electrodes in regions rich with neurons, rather than in fiber bundles is critical. This problem can be mitigated by recording during the implanting process.

The microwire electrode arrays are fixed following surgery; their position cannot be changed. Hence this technique can emphasize the type of information to be gained from observing the same neuron over time. The appearance of similar shaped waveforms on a given wire from session to session is suggestive that the waveform represents the extracellular action potential emitted by one neuron that we are able to follow day after day. Conclusive proof that one is recording the same neuron over prolonged periods is not possible. However, the use of a combination of observations increases confidence that the neuron recorded across two distinct sessions is the same. These factors include the waveform shape, the firing pattern of the unit as indicated by the autocorrelegram, and the average firing rate. Unfortunately, any or all of these parameters may change over time, as a result of plasticity, perhaps especially if a subject's behavior has changed, or as a result of a pharmacological agent, and so conclusions must be drawn with caution.

The longevity of quality recordings within our individual experiments has varied. An example experiment is described in Table 1, which gives the number of sessions for

which neuronal recordings were obtained, and the number of neurons considered to be observed during more than one session. For this study, eight rats were implanted with 8-wire microelectrode arrays into the nucleus accumbens bilaterally (total of 16 wires). The number of sessions for each subject for which good quality recording were obtained varied from one to eighteen. Therefore the possibility exists to obtain a considerable amount of data from one subject. Moreover, long-term studies for the determination of within-subject dose-effect functions or studies with experimental variables that change over time, such as examinations of the acquisition or extinction of a drug habit, become possible.

Over 60% of the accumbens units listed in Table 1 were judged to be the same cells recorded during more than one session. The possibility of testing the same unit under different circumstances over time allows us to ask questions about the stability and specificity of the coding properties of each unit, or of networks of continuously observed units. Figure 1A depicts the activity of an accumbens neuron recorded during performance of an operant task reinforced by 0.1 ml drops of 5% sucrose solution. During the first session depicted here, the solenoid mechanism that delivered the reinforcer was shut off during the middle of the session. The reinforcer was made available once again later in the session, after the subject had ceased to respond. This within-session extinction-reinstatement procedure can be used to examine the sensitivity of some neurons to the presence and absence of the reinforcer during the same session, allowing great confidence that we are observing coding changes for the same neuron. In this case, this unit showed a typical decrease in spike activity at the time of receipt of the reinforcer. This decrease was eliminated when the reinforcer was no longer available and

returned when the reinforcer was again available. Hence this behavioral correlate depends upon the presence of the reinforcer. The stability of the correlate over time can be investigated by looking at the activity of this neuron the following day (Figure 1B), during the performance of the same sucrose-reinforced operant task. We can see that the same decrease in activity during the reinforcer period is present. The autocorrelograms and waveforms for the neuron from each of the two consecutive recording sessions are also shown in Figure 1, C and D. Their similarity in appearance suggests that the neuron is the same.

4. Analysis of Spike Train Data Recorded During Reward-Seeking Behavior

Once the spike trains have been recorded along with the relevant behavioral events, the next formidable task for the electrophysiologist is to determine the meaning of the spike activity in terms of information processing in the brain and in terms of behavior. Issues of neural coding have long been considered by physiologists, psychologists, and neuroscientists. Some decades ago, Perkel and Bullock (1968) provided an exhaustive list of "candidate neural codes or forms of representation of information in the nervous system" that is still relevant today. Their list is comprised of three categories: neuronal events other than impulses, such as post-synaptic potentials, impulses in unit neurons, and ensemble activity. We will be concerned with the latter two categories.

4.1. Single-Neuron Spike Trains

Examination of spike trains recorded in the awake animal reveals a variety of changes related to behavior. When we examine the spike activity recorded from a single neuron in a behaving animal we typically look at changes in firing rate during the behavioral

session and changes in firing rate relative to specific behavioral events such as lever-presses (Figure 2A). Both of these methods have been used to ascribe functions to specific neurons in selected brain regions. In-depth interpretation of these types of neuronal responses is described in the chapter by Peoples (this volume). Determining the type of information encoded in these responses is facilitated by manipulating behavioral variables, as described previously for comparisons of neural coding in the presence and absence of reinforcement. Quantitative evaluations of the relationship between single-neuron firing and specific perceptual and behavioral events have been made and provided important insights in a number a species, including nonhuman primates (please see an early highly influential series of papers from Richmond and colleaugues; Optican & Richmond, 1987; Richmond & Optican, 1987; Richmond et al., 1987), and the book *Spikes* (Reike et al., 1997). An example of this type of analysis as applied to drug abuse research can be found in Bowman et al. (1996). These authors trained rhesus monkeys to respond on a multiple ratio reaction time task for both juice and i.v. cocaine reinforcement during the same session. Principal component and information analyses revealed reinforcer-dependent differences in the way in which accumbens neurons encoded the proximity within the schedule to delivery of reinforcer indicating either that subjects differed in their motivation to work for juice vs. cocaine and/or that natural and drug rewards are encoded differently by accumbens neurons.

4.2. Correlations Between Neuronal Pairs

Within a given brain region, the patterns of activity across networks of neurons are thought to determine the output of that nucleus. The presence of synchronized activity among neurons can be examined by looking at correlated firing between pairs of

simultaneously recorded neurons. In addition, correlated activity can be studied in pairs of neurons from two different anatomically connected regions to characterize the functional relationship of neurons within larger circuits comprised of multiple brain regions. Crosscorrelograms are used to quantify the relationship between the firing of two neurons (Moore et al., 1966). A crosscorrelogram is a histogram that depicts the firing probability, or likelihood, of one neuron relative to a first. The histogram is created by compiling the time intervals between a spike emitted by the reference neuron and each spike emitted from a second target neuron; this process is repeated for all spikes in the reference neuron (see Perkel and Bullock, 1968; Perkel et al., 1967). Hence one obtains a figure depicting the relative likelihood of firing in the second neuron given a spike in the reference neuron (Figure 2B). A flat histogram indicates no relationship between the firing of two neurons. Peaks indicate temporally-related firing; troughs indicate that the second unit is not firing when the first fires.

Neurons might fire in a correlated fashion if they are directly or indirectly synaptically connected, or if they receive common input. With extracellular recording in the mammalian brain it is very difficult to state with certainty that a functional relationship between two units reflects a monosynaptic interaction between the units. Rather, these techniques can be used in the behaving animal to determine functional interactions within and between brain regions to further our knowledge of the dynamics of the neural activity that occurs during behaviors such as drug self-administration. For example, crosscorrelations among neurons in the mesocorticolimbic circuit during i.v. cocaine self-administration have been reported. Chang et al. (2000) found that 18% of neuronal pairs within the nucleus accumbens and the medial prefrontal cortex were

significantly correlated if those neurons that made up the pairs also demonstrated significant phasic activity just before the lever-press made to receive the cocaine infusion (termed, anticipatory activity). In contrast, only 4% of neuronal pairs comprised of neurons without anticipatory activity showed significant correlated activity. This type of finding suggests that interactions among pairs of simultaneously recorded neurons contribute to drug-seeking behavior.

Figure 2B is an example of correlated activity observed within a pair of neurons recorded simultaneously during the operant self-administration of ethanol. The reference neuron in this example was recorded from the medial shell of the accumbens while the target neuron in this pair was recorded from the ipsalateral basloateral amygdala, a region that sends excitatory projections to the medial accumbens. The peak in activity within 200 msec prior to zero indicates that the amygdala neuron tended to fire 200 msec before the accumbens neuron with the greatest likelihood within 50 msec. This finding demonstrates that spike firing within the basolateral amygdala precedes that of accumbens neurons, suggesting that neuronal activity within this region of the amygdala may drive at least a portion of the neuronal activity observed within the accumbens during ethanol self-administration.

Further specificity in the relationship of correlated activity to ongoing behavior can be defined by calculating the crosscorrelation during selected behaviorally-relevant time epochs. For example, Tabuchi, Mulder, and Wiener (2000) calculated crosscorrelations between simultaneously recorded hippocampal and nucleus accumbens neuronal pairs, using only the 1-sec time period prior to reward delivery within a water-reinforced plus maze. These authors found that spiking within hippocampal-accumbens neuronal pairs

tended to be tightly correlated as the subject approached a reward, but not after reward receipt. In this example, crosscorrelation analysis suggests that the anatomical projections from the hippocampus to the accumbens may direct the subject to the location of the reward.

It is possible that correlated activity within a neuronal pair actually reflects coincident responding of both neurons to an external stimulus or event, for instance, to the onset of a conditioned stimulus, rather than an actual physiological relationship between the two neurons. In behavioral paradigms with repeated trials/stimulus presentations, such as operant drug self-administration, this confound can be examined by using a shuffling procedure. This procedure mismatches the real time correlations among two spike trains, and instead looks at the correlated spike firing across all different trials or stimulus presentations, and subtracts the average correlated firing under that condition from the original crosscorrelation statistics. For example, the correlation would be calculated between stimulus presentation 1 for spike train 1 and stimulus presentation 2, 3, 4, and so on, for spike train 2, and then the same is repeated for all other stimulus presentations for spike train 1. The average of all possible shuffles (called the average shift predictor) is subtracted from the original correlation. Any remaining correlation reflects a functional, physiological relationship for the neuronal pair, rather than coincident activity driven by the stimulus alone. This procedure was used by Tabuchi et al. (2000) to examine the hippocampal-accumbens neuronal pairs described above, and the reader is referred to their paper. We can apply this type of analysis to our same neuronal pair from Figure 2D; these results are illustrated in Figure 3A and B. This procedure is important for

disentangling the contribution of coincident activity driven by external stimuli from true functional interactions among neuronal pairs.

4.3. Ensemble Analyses

As our discussion of correlated firing acknowledged, neurons do not work alone; they are embedded in a matrix with thousands of other neurons of the same and different types, and the connections among these neurons shapes their individual firing patterns. Scientists long ago arrived at the conclusion that individual representations (of memories, perceptual events, etc.) must be represented within the brain as a specific pattern of activity distributed across many neurons in space and perhaps time. Quantitative characterization of these patterns in relation to ongoing drug-seeking behavior is a challenge. Researchers have applied traditional multivariate statistical techniques to the examine neuronal ensemble data (Chang et al, 2002; Chapin, 1999; Chapin et al., 1999; Deadwyler et al., 1996; Janak, in press; Laubach et al., 1999, 2000; Nicolelis, 1999; Nicolelis et al., 1999; Wessberg et al, 2000; Stambaugh et al, 2000) as well as statistical techniques specifically tailored for neuronal ensemble analysis such as neuronal network models (Nicolelis et al., 1999), gravity analysis (Gerstein et al., 1985), vector reconstruction techniques (Georgopoulos & Massey, 1988; Georgopoulos et al., 1989), and others (Seidemann et al., 1996). Much more detail on ensemble analyses can be found in *Methods for Neural Ensemble Recordings* (Nicolelis, 1999) or in related publications mentioned above. A simple example is described here to give the reader a sense of the goals, tactics, and problems associated with this approach.

The application of linear discriminant analysis (DA) to ensemble neuronal data is perhaps the simplest of these techniques to understand because it is based upon concepts

we are familiar with from more common statistical procedures such as Analysis of Variance and Multiple Regression. DA is a classification technique that can be used in the context of neuronal ensemble analysis to define different brain states (spatiotemporal patterns of activity) that relate to specific behavioral states (events). DA attempts to find the best linear combination of weighted variables that will separate experimental categories by maximizing the between-group variance relative to the within-group variance (Bray & Maxwell, 1985). This kind of analysis works for experimental designs based upon repeated occurrences, such as trials, found within operant self-administration paradigms. To conduct this type of analysis, each variable is an array of values that represents the spike firing of a given neuron, usually within a given time bin, with each value representing the spike firing for one trial. Each case, then, represents the spike firing across all variables (neurons) for a given trial.

An illustration of a question we might ask is whether accumbens ensemble neural activity that occurs around the time of the operant response is sensitive to the presence or absence of the reinforcer. As an example, let us say we obtained simultaneous recordings from 10 accumbens neurons from a subject whose operant responding was reinforced for the first half, but not for the second half, of the experimental session (within-session extinction). We've recorded ensemble spike activity from trials for which the operant response was and was not followed by reinforcer. DA will allow us to test whether we can discriminate between the pattern of activity within the ensemble on trials that were or were not reinforced. The data preparation and anlaysis steps are described briefly here and in Figure 4. Let us say we recorded from these 10 neurons across 100 trials (50 trials were reinforced, 50 trials were not reinforced), and we want to examine the 2 second time

period around the operant response. We now need to choose a relevant time bin for analysis within this 2 second time period. One might do this by trial and error, or by examination of the single-neuron perievent histograms. If we chose 500 msec time bins, we would have 40 variables in our analysis (4 bins x 10 neurons). A DA would then find the weighted combination of all variables that would maximally discriminate between cases (trials) that were reinforced (Condition 1) and that were not reinforced (Condition 2). Some of the cases are used to build the model, and other cases are used to test the model, to control for over-fitting the data. If the analysis is not able to classify the individual test trials with any likelihood greater than chance then it is possible that the ensemble spike firing that occurs when the subject performs the operant response is not sensitive to the outcome of the operant response. Classification better than chance might indicate that our ensemble does contain information about the outcome. The analysis can be used to determine the relative importance of individual neurons to the performance of the entire ensemble. Likewise, if an ensemble of neurons performs better than any one neuron, then we have strengthened the notion that it is networks of neurons, not individual neurons, that are the functional units that carry information in the nervous system. Of course, like many statistical techniques, the appropriate application of DA to any data set is based upon certain assumptions, and therefore it is critical that attention be paid to these aspects of the analysis to avoid false positive outcomes (see Chapin, 1999 and Bray & Maxwell, 1985 for more information).

Analyses like DA are informative for these early stages of interpreting spatiotemporal patterns of neural activity, but of course are necessarily based upon assumptions about the underlying biology. For example, by choosing a certain bin width for the data we are

assuming that we have a relevant unit of measure to capture the phenomenon in which we are interested. If our time bins are too big, one might lose the fine grain necessary to sensitively detect certain interactions among neurons relative to ongoing behavior. However, if our time bins are very small we are increasing the total number of variables in the analysis, contributing to a decrease in statistical power and a possible increase in highly correlated (redundant) variables that contribute to spurious results (Bray & Maxwell, 1985). In addition, by defining a small number of behavioral states a priori we are undoubtedly oversimplifying the explanation of patterns of neural activity. Techniques of analysis that are unsupervised may provide more biological relevance by allowing structure to be determined within the data empirically.

3. Future Directions

Advances in the technique of many-neuron recording are likely to continue and result in increased numbers of neurons that can be simultaneously recorded. Such advances will likely include changes in microelectrode design, in the on-line data sampling capabilities of our hardware, and in the computational power for storing and analyzing acquired data. These technical advances will allow us to continue to refine the precision of our view of real-time brain function in the awake behaving animal.

In the future it is likely that increasing numbers of experiments will combine current techniques to provide a powerful means to address specific issues within the study of the neurobiological basis of drug self-administration. Pharmacology, neurochemistry, and molecular biology can all be combined with neurophysiology. For example, many-neuron recording can be conducted in genetically-altered mice to understand better the

means by which specific genes may alter the neuronal activity that drives drug self-administration.

The most important obstacle for many-neuron recording is not a difficulty but a challenge -- in fact it is the essential scientific problem in behavioral neurophysiology. This problem is one of interpretation of the neural data we record. We now have access to the actual millisecond-by-millisecond activity of networks of neurons in the awake functioning brain – but what does it all mean? Perhaps we are able to read the letters but we're still struggling to understand the meaning of the words. There is much work to be done to decipher the information that we are now able to record from behaving animals. Understanding the means by which neuronal networks function to control addictive behavior, and all behavior in general, will be an exciting undertaking for the years to come.

6. Conclusions

Advances in our ability to acquire and store digital information now allow us to sample from large numbers of neurons simultaneously throughout the brain. It appears that the sampling rate is sufficient to permit a faithful record of the spike output of large ensembles of neurons, allowing us to examine in detail the relationship among individual neurons within an ensemble and between these neurons and the behavior of the animal. The pursuit of the nature of neural encoding and function that controls drug-seeking will provide fundamental understanding of the neurobiological basis of addictive behavior.

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Figure Legends.

Figure 1. Stability of Recording in Behaving Rats. Accumbens neurons were recorded during the performance of a nosepoke response reinforced by a 5% sucrose solution. A. Three perievent histograms that depict the average firing rate of one unit recorded from the nucleus accumbens during the operant self-administration of sucrose. In this session, the subject performed 35 trials that were reinforced ('Reward Self-Administration'), an additional 23 trials that were not reinforced ('Extinction'), and a further 35 trials that again were reinforced ('Reward Reinstatement'). Histograms are aligned at the time at which the 0.1 ml drop of sucrose was delivered (t=0 on the x-axis). A reward-related decrease in spike activity is apparent during Reward Self-Administration that is absent during Extinction. When the sucrose is again delivered following an operant response, the decrease in spike activity is once more observed ('Reward Reinstatement'). B. A perievent histogram depicting the average response of the same neuron as in 'A' recorded during the next day's session in which the subject performed 47 reinforced trials. A decrease in spike activity following reward receipt is again observed. C. Autocorrelogram and waveform (inset) for same neuron as in 'A'. The autocorrelogram depicts the spike activity of the unit during the entire behavioral session as a function of the {1^{st} order, 2^{nd} order...n^{th} order} time intervals between each reference spike and the remainder of the spikes in the spike train. Hence the autocorrelogram depicts the firing pattern (tonic vs. phasic) of the unit. D. Autocorrelogram and waveform for same unit as in 'B'. The similarity between the autocorrelograms and waveforms in 'C' and 'D' suggest that they are the same unit.

Figure 2. Spike Train Analysis.

A. Single Neuron Analysis. 1. *Rate histograms.* These histograms allow observation of changes in the average firing rate or frequency (Hz) of one neuron as conditions change during the recording session. In the example presented here, the experimental manipulation at time = 700 sec was followed by an overall increase in firing frequency by this unit. 2. *Rasters.* Rasters, or spike trains, depict the time of occurrence of each spike recorded from a given neuron. Patterns in the spike train may be apparent. In this case, a comparison of the time of occurrences of an experimental event, such as a leverpress, with the spike train indicates that the unit bursts prior to the emission of the operant response. 3. *Perievent histograms and rasters.* To better determine the relationship between a spike train and a repeated behavioral event, a perievent histogram is created. The x-axis is time relative to each the behavioral event, such as a leverpress and the y-axis is firing frequency (Hz). The average firing frequency of one neuron relative to all instances of the experimental event is depicted. In this example, the unit increased spiking just prior to the occurrence of the event. The raster, above, depicts the spike activity (spike train) of the neuron for each individual instance (trial) of the behavioral event, aligned on the same time scale as the perievent histogram. Thus, the perievent histogram is the average of the spike activity across all of the rasters.

B. Neuronal Pairs. 1. *Two Simultaneously Recorded Spike Trains.* To detect the presence of non-random relationships between the firing of two simultaneously-recorded neurons using crosscorrelograms, the cross-neuron inter-spike intervals are tabulated. For each spike in the spike train for the reference neuron, all possible interspike intervals between that reference spike and each spike in the spike train of the target neuron are

determined. 2. *Crosscorrelogram.* The cumulated intervals determined for all possible inter-spike intervals between the reference and target neurons are displayed in a histogramn following conversion of the units to either firing probability or frequency; t=0 on the x-axis corresponds to the time of occurrence of each spike in the reference neuron. In this example, the y-axis shows the firing frequency of the target unit relative to a reference neuron spike. The horizontal dotted line is the 95% confidence interval; bars above this dotted line represent significantly correlated firing between the two units. For this neuronal pair, the target amygdala neuron tends to fire at 4 Hz within the 50 msec interval preceding a spike in the reference accumbens neuron.

Figure 3. Event-related Crosscorreleograms.

A. A crosscorrelation between the same neurons in Figure 2.B.2. calculated only during the 2-second time interval following delivery of the reward. No correlation is present between the two units in the 2-second interval prior to reward delivery (data not shown).

B. Shift predictor histogram produced following shuffling procedure on same data as in A. A significant correlation between the two neurons still exists following subtraction of shuffled data, as revealed by peaks above the 95% confidence interval line, suggesting that the correlated firing is physiological.

Figure 4. Steps in Discriminant Analysis of Neuronal Ensemble Data. A. Spike train data is tabulated into time bins relative to a behavioral event. In this example, the average firing rate relative to the performance of the operant response (t=0 on the x-axis) is depicted, with a 500-msec bin size. A 2-second time period around the operant

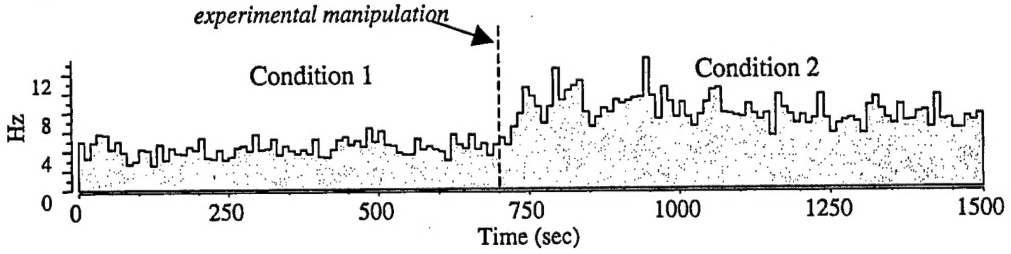
response is chosen for analysis. B. The spike activity of each neuron in each of the 4 500-msec time bins that surround the operant response is tabulated for each trial. Thus, each vertical column in the spreadsheet represents one time bin for one neuron (variable). Each horizontal row in the spreadsheet represents all the data from one trial (case). The categorization variable on the far left, Condition, indicates the type of trial by using a numerical code. C. The spreadsheet is imported into an analysis program such as SPSS for step-wise discriminant analysis. This procedure determines if our variables can be used to discriminate between our two trial types and, if so, which variables contribute to the model. In this example, nine variables from 6 neurons were included in the model. The coefficient calculated for each variable is shown. A discriminant score for each trial case (trial) can be calculated by multiplying the value of the included variables for that trial by their respective coefficients. The frequency histogram of the discriminant scores obtained for each trial finds little overlap between the two trial types, indicating good discrimination. In this example, the model correctly classified the trial type for 91.8% of the trials (50% is chance).

Table 1. Typical Accumbens Neuronal Yields From Bilateral 8-wire Microarray Electrodes: A Representative Study

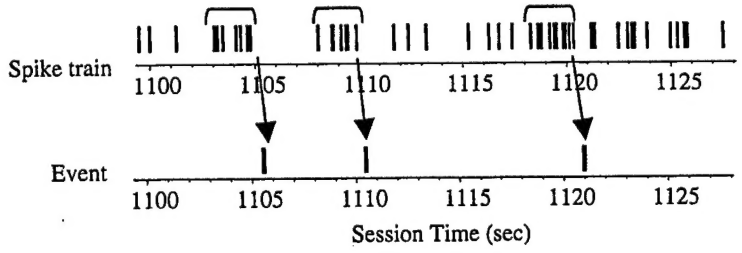
			Number of Neurons Recorded		
	Subject	**Number Sessions Analyzed**	**In Only One Session**	**In More Than One Session**	**Total Units**
	1	1	12	0	12
	2	5	11	9	20
	3	5	12	12	24
	4	13	16	9	25
	5	18	0	19	19
	6	14	0	9	9
	7	14	3	12	15
	8	15	1	9	10
Totals	*8*	*85*	*55*	*79*	*134*
mean		*10.63*	*6.85*	*9.88*	*16.75*
SD		*6.07*	*6.51*	*5.25*	*6.18*
SEM		*2.15*	*2.30*	*1.86*	*2.19*

A Single-Neuron Analysis

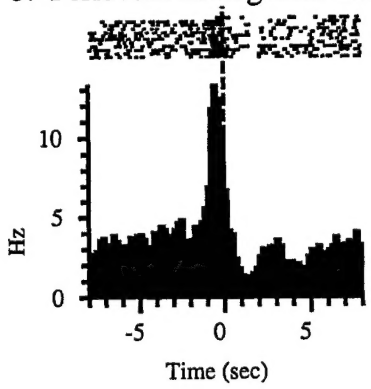
1. Rate Histograms



2. Rasters

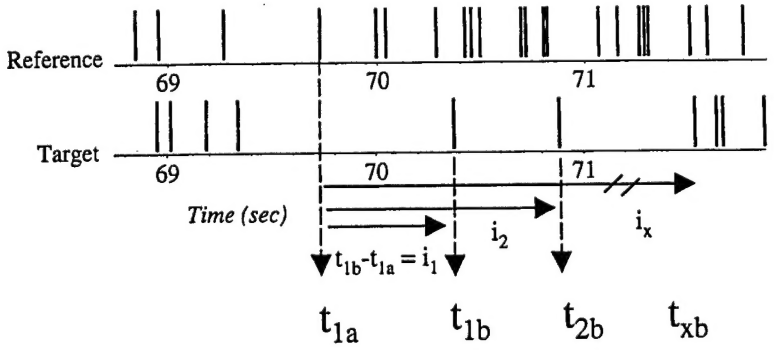


3. Perievent Histograms and Rasters

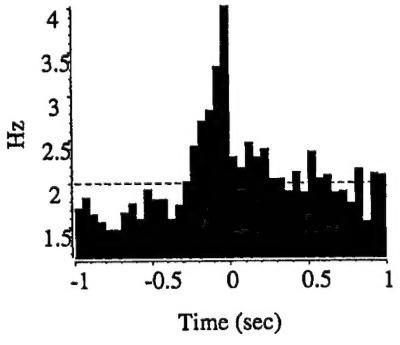


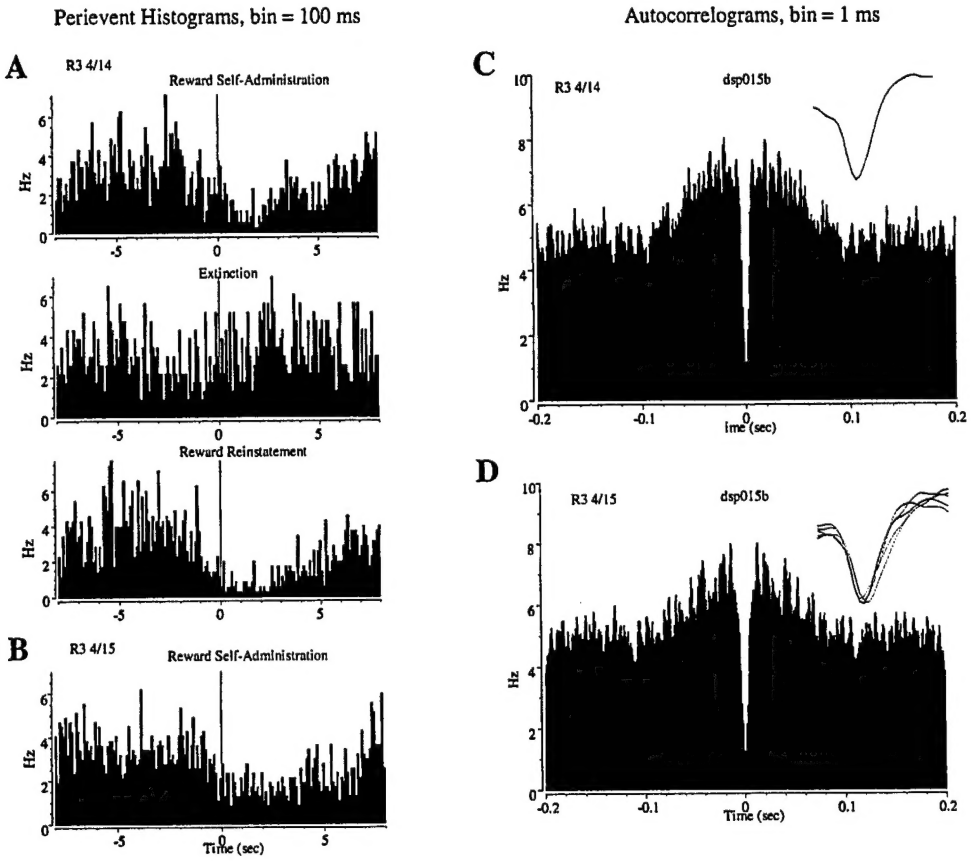
B Neuronal Pairs

1. Two Simultaneously Recorded Spike Trains



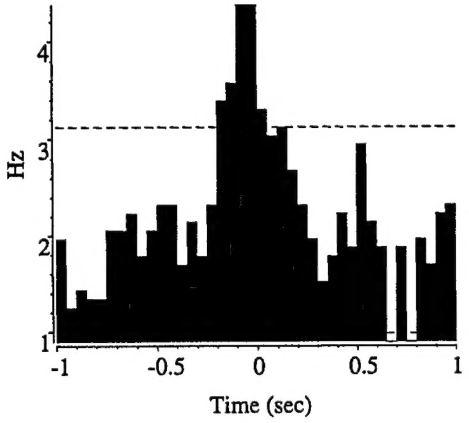
2. Crosscorrelogram




Perievent Histograms, bin = 100 ms
Autocorrelograms, bin = 1 ms
A
R3 4/14
Reward Self-Administration
Extinction
Reward Reinstatement
B
R3 4/15
Reward Self-Administration
Hz
Time (sec)
C
R3 4/14
dsp015b
D
R3 4/15
dsp015b

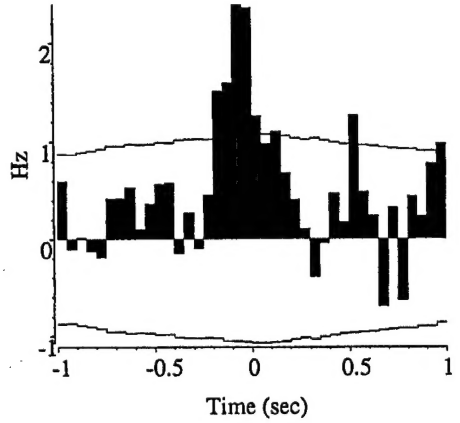
A

Crosscorrelogram During 2-s Reward Period



B

Shift Predictor

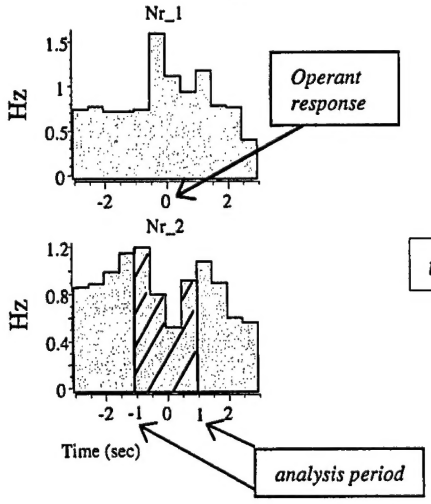


Linear Discriminant Analysis

A

Bin spike counts.
500 msec bin size.

Perievent Histograms



B

Create trial-by-trial spreadsheet.
Variables are columns of spike activity for one neuron during one time bin for every trial. The classification variable indicates the conditions (trial type).

neurons →

trials ↓

Condition	1_bin1	1_bin2	1_bin3	1_bin4	2_bin1	2_bin2	2_bin3	2_bin4	3_bin1	3_bin2	...
1	1	2	1	1	1	1	0	0	2	1	...
1	2	1	0	2	1	2	1	0	0	0	...
1	1	0	2	4	0	0	1	0	3	3	...
2	3	2	1	3	2	1	1	1	0	1	...
2	1	1	1	1	1	0	2	0	2	0	...
2	3	1	1	3	0	1	2	0	0	1	...
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	

C

Conduct stepwise linear discriminant analysis. 40 Variables entered. Variables remaining in analysis along with their discriminant function coefficients (c_x):

Variables	*c*
N02A06	-.783
N03A04	-.609
N04A03	.576
N04A05	.572
N05C06	1.111
N05C07	1.105
N06A06	.567
N08A02	1.016
N08A06	.694

Discriminant Function:
For each case,
Discriminant score = $c_1x_1 + c_2x_2 \ldots c_9x_9$

Discriminant Scores by Condition